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The Potency of Secondary Metabolites Endophytic Fungi *Trichoderma* sp as Biocontrol of *Colletotrichum* sp and *Fusarium oxysporum* Causing Disease in Chili

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Abstract. In the cultivation of chili plants, there are some pathogen fungi that often attack chili plants and can reduce productivity. The pathogenicity of the genus *Colletotrichum* sp. is so strong that it can reduce chili production in quantity and quality, the disease called antracnose diseasse. *Fusarium oxysporum* mold can also reduce the productivity of chili. *Trichoderma sp.* is known to have a major role in controlling plant diseases. The secondary metabolites produced by *Trichoderma sp.* play an important role in its antifungal activity. This research aims to find endophytic fungi *Trichoderma* sp which are potential as a biocontrol agent against *F. oxysporum* causing wilt disease in chilli. In this study, the antifungal activity of volatile and non-volatile secondary metabolites of endophytic fungi *Trichoderma* sp. was tested against *F. oxysporum* and *Colletotrichum* sp. Antifungal activity of secondary volatile endophytic fungi *Trichoderma sp.* is able to inhibit *Colletotrichum sp.* growth by 41.11% and *F. oxysporum* at 12.45%. While non-volatile metabolites with a concentration of 30% displayed the greatest ability to inhibit the growth of *Colletotrichum sp.* by 20.58% and *F. oxysporum* by 13.02%.

INTRODUCTION

Chili is one of the horticultural commodities that is widely cultivated by farmers and also widely consumed by people in Indonesia, both on household and industrial scale. One of the problems in chili cultivation is the ever-present threat of pests and disease attacking the plants since planting until harvest time. The disease often found in chili is anthracnose caused by the pathogen *Colletotrichum sp.* [1]. The pathogenicity of the genus *Colletotrichum sp.* is so strong that it can reduce chili production in quantity and quality [2]. The *F. oxysporum* fungi can also reduce the productivity of chili by 50% [3]. One of the attempts commonly implemented to prevent the attack of pathogenic fungi is the use of synthetic fungicides. However, excessive use of synthetic fungicides gives negative impacts on human and the environment, among other things is the remaining fungicide residues in the soil which threaten to poison non-target organisms and get carried over to water sources, thus poisoning the surrounding environment. Fungicide residues in plants might harm those who consume them, whether it is animal or human [4].

The use of biological control such as *Trichoderma sp.* as a biocontrol agent is one of the alternative methods. *Trichoderma spp.* is deemed highly potential as biocontrol agent because of its rapid growth and easily-cultured nature, both in culture medium and natural conditions. Several types of *Trichoderma spp.* including *T. harizanum*, *T. viridae*, *T. virens*, *T. hamatum*, *T. roseum*, and *T. koningii* are species often used as a biological control agent [4].

The secondary metabolites produced by *Trichoderma* sp. play an important role in its antifungal activity [5]. Secondary metabolites can be an elicitor that functions in resistance plants against attacks by pathogenic organisms [6]. *Trichoderma spp*. produces secondary metabolites in the form of antibiotic compounds, enzymes, toxins, and hormones. Antibiotic compounds produced by *Trichoderma spp.* among them are viridins (produced by *T. viriens*), kininginins (produced by *T. koningii*), cytosperone, trichodermol, mannitol, and 2-hydroximalonate acid. The

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secondary metabolites of *T. viride*, *T. atroviride*, *T. harzianum*, and *T. koningii*, namely 6-pentyl-yr pyrone, have been used to control plant diseases caused by fungi [6-7].

Environmental friendly biocontrol agents are widely applied to solve the problems of plant pests and diseases, such as the use of endophytic *Trichoderma spp*. Endophytic fungi are fungi found in plant tissue systems including leaves, flower, twig and root system. Endophytic fungi grow and feed on their host plants and can infect healthy plants in certain tissues, capable of producing an assortment of mycotoxins, enzymes and antibiotics [8]. The objective of this study was to determine the potential of secondary metabolites endophytic fungi *Trichoderma sp*. as a biocontrol agent of *Colletotrichum sp.* and *F. oxysporum* causing disease in chili in vitro.

EXPERIMENTAL DETAILS

Fermentation of Endophytic Fungi *Trichoderma sp.*

Endophytic fungi *Trichoderma sp*. used were isolated from strawberries which are a collection of UIN Malang microbiology laboratories. Sporulated endophytic fungi was cut into three pieces of ± 1 cm x 1 cm and were inoculated into 100 ml Potato Dextrose Broth (PDB) media in 250 ml Erlenmeyer flask. The cultures were fermented in a rotary shaker at 150 rpm with a temperature of 28°C [9]. The fermentation process was carried out for seven days, in compliance with the stationary phase based on the growth phase of endophytic fungi *Trichoderma sp.* (unpublished data).

Anti-fungal Activity Assessment of Endophytic Fungi *Trichoderma sp.* Volatile and Non-volatile Metabolites

Anti-fungal activity assessment of volatile metabolites was initiated by filtrating the result of fungal fermentation using Whatman No. 1 filter paper. The filtrate was then centrifuged at 12,000 rpm for 20 minutes. The supernatant was taken and then filtered with 0.2 µm (millipore) stringer, followed by pasteurization at 60ºC for 30 min [10]. The filtrate was inserted into PDA media which have not yet solidified (temperature 45ºC) at concentrations of 10%, 20%, and 30%. The treatment without filtrate serves as negative control while positive control was prepared using propinep 70%. After the medium solidified, pathogen fungi were inoculated at the center of PDA medium, 6 mm in diameter. The culture was then incubated at 28°C for seven days [6]. The parameters observed were the diameter of pathogenic fungi and the inhibition percentage.

Anti-fungal activity assessment of non-volatile metabolites was carried out by growing endophytic fungi *Trichoderma sp.* and pathogenic fungi on PDA medium in a petri dish. After three days, the lid was lifted off and the plate containing endophytic fungi and pathogenic fungi were staked together with the endophytic fungi at the bottom. The plate was affixed using three parafilm layers and then incubated for seven days at 28°C. Petri dish containing PDA without endophytic fungi was prepared as a control treatment. The treatment was carried out in tree replications and observed every day for seven days [6]. The parameters observed were the colony diameter of the pathogenic fungi and the inhibition percentage

Inhibition percentage of endophytic fungal secondary metabolites on the growth of pathogenic fungi was determined using the following equation [11]:

$$\text{P (\%)} = \frac{(DK-DP)}{DK} x\ 100\% \quad (1)$$

Note: P = growth inhibition percentage of pathogenic, DK = colony diameter of pathogenic fungi on control culture, DP = colony diameter of pathogenic fungi on treatment culture.

RESULTS AND DISCUSSION

Antifungal Activity of Volatile and Non-Volatile Secondary Metabolites of Endophytic *Trichoderma sp.*

The results of the study showed that volatile secondary metabolites of endophytic fungi *Trichoderma* sp. are able to inhibit *Colletotrichum* sp. growth by 41.11% and *F. oxysporum* growth by 12.45% (Table 1 and Fig. 1). Volatile secondary metabolites produced by *Trichoderma sp*. exhibit high potential as anti-fungal [12]. Vargas *et al.* [13] stated

that a large variety of volatile secondary metabolites could be produced by *Trichoderma* spp. such as hydrogen cyanide, ethylene, aldehydes, and ketones, which an important role in controlling various plant pathogens.

This is presumably due to the presence of volatile secondary metabolites produced by *Trichoderma* sp. which are capable of affecting the growth of pathogenic fungi. These secondary metabolites play a key role in mycoparasites and their interactions with the plants.

Jelen *et al*.[14] stated that among volatile antifungal compounds produced by *Trichoderma sp*., the most important and well-documented was 6-pentyl-a-pyron (6-PAP), a polyketide with an aroma resembling sweet coconut that has been reported to have antimicrobial activity and herbicides. Additionally, Jelen *et al* [14] reported eight isolates of *Trichoderma* sp. including *T. atrovirid*, *T. citrinoviride*, *T. hamatum*, *T. harzianum*, *T. koningii*, and *T. viride* exhibited the capability of producing 6-n-pentyl-2H-pyran-one compounds (6-PAP), that plays a role in the inhibition of pathogenic *F. oxysporum*.

TABLE 1. Antifungal activity of volatile secondary metabolites from endophytic fungi *Trichoderma* sp.

Treatment	Average Diameter of Colony (cm)	Average Inhibition (%)
Colletotrichum sp.+*Trichoderma* sp.	2.65	41.11± 2.40
Colletotrichum sp.(control)	4.50	
Fusarium oxysporum +*Trichoderma* sp.	6.34	12.45 ± 3.36
Fusarium oxysporum (control)	7.25	

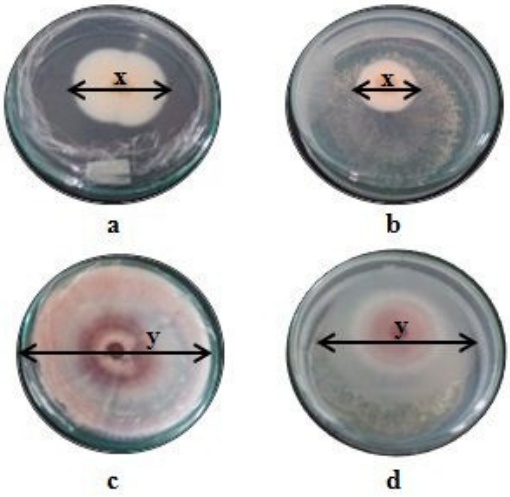

FIGURE 1. Pathogen colonies on day 7 of antifungal activity assessment of volatile secondary metabolites (a) *Colletotrichum* sp. (control), (b) *Colletotrichum* sp. (treatment); (x) is colony diameter of *Colletotrichum* sp., (c) *F. oxysporum* (control), (d) *F. oxysporum* (treatment); (y) is colony diameter of *F. oxysporum*.

The results of antifungal activity assessment on non-volatile secondary metabolites of endophytic fungi *Trichoderma* sp. exhibited an effect on colony diameter and growth inhibition of *Colletotrichum* sp. and *F. oxysporum* (Table 2 and Fig. 2). Based on Table 2, the higher the concentration given the smaller the diameter of *Colletotrichum* sp. and *F. oxysporum* resulting in the greater inhibition given. But based on statistical analysis, the difference in concentration treatment given to *Colletotrichum* sp. has the same notation b, which means that the concentration of 10%, 20%, and 30% is not significant or not significantly different. Whereas when compared to negative control treatment (K-) and positive control treatment (K+) have different notations which means there are significant differences or significantly different.

Based on the results, non-volatile metabolites with a concentration of 30% displayed the greatest ability to inhibit the growth of *Colletotrichum* sp. by 20.58% and *F. oxysporum* by 13.02%, however it is still considerably low compared to the positive control (K+) using propinep 70% of *Colletotrichum* sp. by 39.16 % and *F. oxysporum* by 33.02 %. The influence of the non-volatile metabolite endophytic fungi *Trichoderma* sp. against *Colletotrichum sp* and *F. oxysporum* shows that the presence of compounds that can inhibit the mold pathogen. It is also reported that harzianolide compounds produced by *T. harzianum* can inhibit the germination of *F. oxysporum* spores [15].

TABLE 2. Antifungal activity of non-volatile secondary metabolites from endophytic *Trichoderma* sp. against pathogenic fungi.

Treatment	Average Diameter of Colony(cm)		Average Inhibition (%)	
	***Colletotrichum* sp.**	***F. oxysporum***	***Colletotrichum* sp.**	***F. oxysporum***
0% (K-)	5.08 ± 0.25^{c}	8.06 ± 0.14^{d}	0 ± 0^{a}	0 ± 0^{a}
10%	4.48 ± 0.19^{b}	7.55 ± 0.10^{c}	11.46 ± 3.84^{b}	6.36 ± 1.31^{b}
20%	4.26 ± 0.12^{b}	7.53 ± 0.21^{b}	16.80 ± 2.45^{b}	6.67 ± 2.72^{b}
30%	4.15 ± 0.12^{b}	7.01 ± 0.27^{b}	20.58 ± 2.51^{b}	13.02 ± 3.37^{c}
K+	3.09 ± 0.33^{a}	5.40 ± 0.18^{a}	39.16 ± 6.65^{c}	33.02 ± 2.32^{d}

Note: Identical letter notations show that the differences between the results are not significant based on the 5% DMRT test.

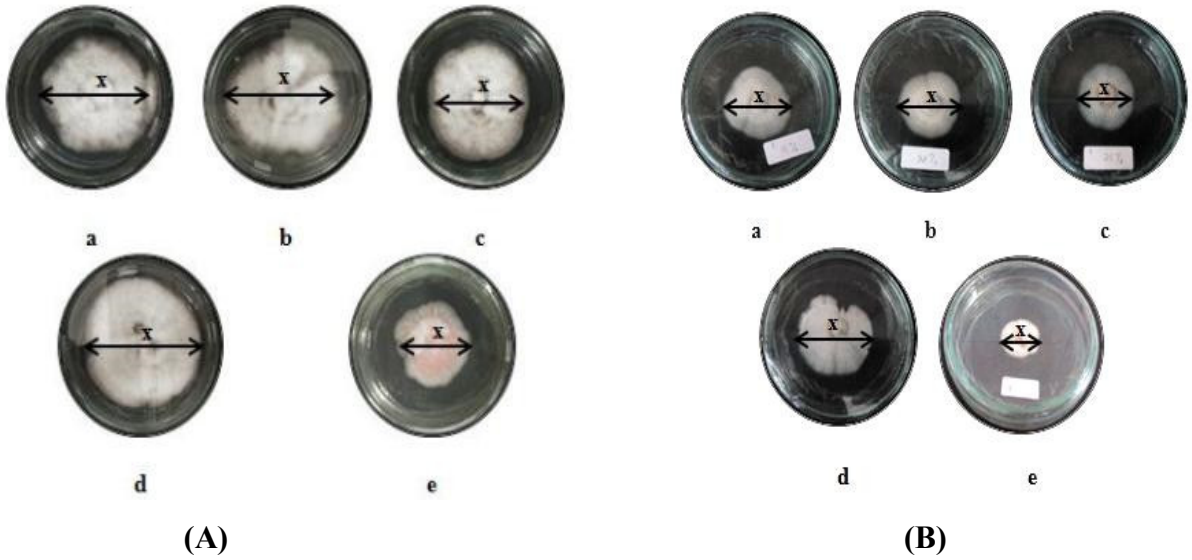

FIGURE 2. Colonies of pathogenic (A) *F. oxysporum* ; (B) *Colletotrichum* sp. on day 7 of anti-fungal activity assessment with the treatment of non-volatile metabolites endophytic fungi *Trichoderma* sp. of (a) 10%, (b) 20%, (c) 30% concentration, (d) negative control and (e) positive control; (x) is colony diameter of pathogenic fungi

The observation results on the effect of volatile metabolites produced by endophytic fungi *Trichoderma* sp. to the hyphal growth of pathogenic *F. oxysporum* showed distinctive macroscopic differences between the aerial hyphae of pathogenic fungi with and without treatment of volatile metabolites. Fig. 3 shows that the aerial hyphae of *F.oxysporum* treated with volatile metabolites of endophytic fungi *Trichoderma* sp. experienced alteration or become abnormal. According to Mukherjee *et al*. [7], secondary metabolites with antibiotic activity have the ability to enter fungal cells and causing mycolysis, which is the loss of protoplasm in cell wall structure preventing the dissolution of the enzyme in the cell wall of the mold. Micolysis leads to a number of symptoms including swelling, shortening and cell wall lysis, thus resulting in abnormal hyphal growth.

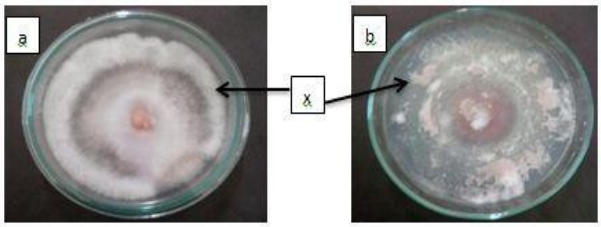

FIGURE 3. Macroscopic observation on pathogenic *F. oxsporum* fungi with the treatment of volatile metabolites from endophytic fungi *Trichoderma* sp. on day 7 (a) *F. oxysporum* (control) (b) *F. oxysporum* (after treatment); (x) is the aerial hyphae of *F. oxysporum* fungi colony.

SUMMARY

Antifungal activity of secondary volatile endophytic fungi *Trichoderma* sp. able to inhibit *Colletotrichum* sp. growth by 41.11% and *F. oxysporum* at 12.45%. While non-volatile metabolites with a concentration of 30% displayed the greatest ability to inhibit the growth of *Colletotrichum* sp. by 20.58% and *F. oxysporum* by 13.02%.

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